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Ca^{2+}-DEPENDENT ACTIVATION OF PHOSPHORYLASE BY PHOSPHORYLASE KINASE IN ADIPOSE TISSUE

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Summary

Phosphorylase kinase (EC 2.7.1.38) activity in crude cytosol preparations of chicken adipose tissue was assayed using as substrate either the endogenous phosphorylase *b* in the preparation or added purified rabbit skeletal muscle phosphorylase *b*. The results obtained with the two substrates were similar. The phosphorylase kinase reaction was markedly inhibited by ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), maximum inhibition (about 90%) occurring at approx. 0.2 mM. This inhibition was readily reversed by addition of Ca^{2+}. Full reversal was achieved with 0.3 mM Ca^{2+} in the presence of 0.5 mM EGTA; the estimated free Ca^{2+} concentration required was 4 μM. The activation of phosphorylase *b* was blocked immediately and completely by EGTA added during the course of the assay; reversal was achieved without a time lag by the addition of Ca^{2+}. The Ca^{2+} requirement was also demonstrated directly by preparing an enzyme fraction from which Ca^{2+} had been removed and by using Ca^{2+}-free reagents. Under these conditions the Ca^{2+} concentration needed for half maximum activation was 10 μM and maximum activation was obtained at about 100 μM. The possibility that the effects of EGTA and Ca^{2+} might be related to changes in phosphorylase phosphatase activity rather than phosphorylase kinase was considered unlikely since the phosphorylase phosphatase activity was inhibited during the phosphorylase kinase assay step by the inclusion of fluoride and β-glycerophosphate. Phosphorylase kinase activity in rat adipocytes, using endogenous phosphorylase as substrate, was also inhibited EGTA but, whereas the activity in chicken adipose tissue was inhibited by 90%, the activity in rat adipose tissue was inhibited only 60%. These data indicate that adipose tissue phosphorylase kinase has a Ca^{2+} requirement for optimal activity and is thus qualitatively similar to the enzyme in contractile tissues.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

Introduction

Activation of phosphorylase *b* by phosphorylase kinase (EC 2.7.1.38) in rabbit skeletal muscle requires micromolar concentration of Ca^{2+} as demonstrated by Ozawa et al. [1] and was later examined in greater detail by Brostrom et al. [2]. Phosphorylase kinase from dog, rabbit cardiac muscle and chicken gizzard smooth muscle [1,3], chicken breast muscle [4], insect flight muscle [5], and guinea pig brain [6] have also been shown to require Ca^{2+} at micromolar concentrations.

We have previously reported that activation of rabbit skeletal muscle phosphorylase by crude supernatant fractions prepared from rat adipocytes required Ca^{2+}, being inhibited by 60% in the presence of 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) [7]. On other hand, the activation of endogenous adipose tissue phosphorylase did not appear to be inhibited by EGTA under similar conditions. Recently, we have shown that the hormone-sensitive lipase of chicken adipose tissue is activated more strikingly by cyclic AMP-dependent protein kinase than that of rat adipose tissue [8]. In connection with the studies of the mechanism of activation of hormone-sensitive lipase we have carried out a more systematic investigation of the activation of phosphorylase. The results reported here show that phosphorylase kinase activity in chicken adipose tissue is almost completely dependent upon Ca^{2+}. Reinvestigation of phosphorylase kinase activity in rat adipocytes shows that, while it has some activity in the absence of Ca^{2+}, addition of Ca^{2+} further enhances activity by almost 100%. A preliminary report of these findings has been published [9].

Materials and Methods

Preparation of enzyme fractions

Laying hens (White Leghorn) were killed by decapitation and adipose tissue was dissected from the abdominal region and around the crop. The fresh tissues were minced and homogenized for 30 s at 10—15° C in a Waring blender with 2 volumes of a buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris · HCl, pH 7.4. The homogenate was centrifuged at low speed (5000 × *g*) for 5 min to remove the bulk of the fat cake and the infranatant fluid was filtered through glass wool and centrifuged at 100 000 × *g* for 60 min. Floating fat was drawn off and the fluid was again filtered through the glass wool. Because phosphorylase kinase activity in this fraction was very low, it was concentrated (30-fold) by precipitation at pH 5.2. Acetic acid (0.2 M) was added in an ice bath with constant stirring to adjust pH; after 5—10 min, the precipitate was collected by centrifugation at 1000 × *g* for 10 min. The precipitate was resuspended and homogenized in a small volume of homogenizing buffer containing no EDTA. It was adjusted to pH 7.4 and could be stored at −80° C for as long as 1 month without loss of phosphorylase kinase or phosphorylase activity. The phosphorylase activity ratio in this fraction (assayed in the absence and in the presence of 2 mM AMP) was as low as 0.04; on incubation with ATP-Mg^{2+} for 5 min at 30° C, the phosphorylase activity ratio rose close to 1.0. This pH 5.2 precipitate fraction was the enzyme preparation

used in all the experiments to be described.

Rat adipocytes were isolated from epididymal fat pads according to Rodbell [10]. The pH 5.2 precipitate fraction was prepared as described above.

Enzyme assays

Phosphorylase kinase assay was carried out in two steps, a preincubation under conditions appropriate for conversion of phosphorylase *b* to phosphorylase *a* and a subsequent assay of phosphorylase *a* activity. The standard phosphorylase kinase reaction mixture consisted of 50 mM β-glycerophosphate, 10 mM KF, 13 mM β-mercaptoethanol, 15.5 units/ml creatine kinase, 5 mM phosphocreatine, 1 mM ATP, 5 mM $MgCl_2$, 0.2 mg of the enzyme fraction to be assayed and approximately 240 units/ml of purified skeletal muscle phosphorylase *b* at pH 6.8 in a final volume of 50 μl. In some experiments, no phosphorylase *b* was added and only the endogenous phosphorylase *b* in the preparation was used as substrate.

The phosphorylase *a* formed in the first step was assayed in the direction of glucose-1-*P* formation. When endogenous phosphorylase *b* was used as substrate, a 5-μl aliquot from the phosphorylase kinase reaction was transferred immediately to 50 μl of a standard phosphorylase assay mixture which consisted of 50 mM phosphate, 10 mg/ml glycogen and 0.1 mg/ml bovine serum albumin at pH 6.8 and assayed for 30 min at 30°C during which time the reaction was linear. When exogenous phosphorylase *b* was used as substrate, phosphorylase kinase was assayed by a modification [11] of the methods of Krebs et al. [12]. 5'-Nucleotidase (1.6 units/ml) was included in the phosphorylase *a* assay to reduce any possible effects of AMP generated from ATP or present as a contaminant in the assay mixture. The rate of glucose-1-*P* formation was measured using a coupled enzyme reaction (phosphoglucomutase plus glucose-6-phosphate dehydrogenase) and measuring the NADPH formed fluorimetrically [13]. One unit of phosphorylase was defined as the enzyme activity yielding 1 μmol of glucose-1-*P* formed per min under standard assay conditions. The results were expressed as milliunits/mg protein with endogenous phosphorylase *b* as substrate, and as units/mg protein with exogenous phosphorylase *b* as substrate.

Phosphorylase phosphatase activity was measured by incubating the pH 5.2 precipitate fraction with an excess of purified rabbit skeletal muscle phosphorylase *a* in a 50 mM Tris · HCl buffer, 13 mM β-mercaptoethanol, pH 7.5, in a total volume of 50 μl. The reaction was terminated by 20-fold dilution into the phosphorylase *a* assay mixture as described for phosphorylase kinase assay with exogenous phosphorylase *b*. Phosphorylase activities were assayed in the absence and in the presence of 2 mM AMP. The initial phosphorylase activity ratio was close to 1.0. The rate of decrease in activity ratio was taken as a measure of phosphorylase phosphatase activity.

Removal of Ca^{2+} from reagents and enzyme preparations

Chelex 100 (Bio-Rad Laboratories), a resin specific for chelating divalent cations, was used to remove Ca^{2+} from reagents. The chelating ability of Chelex 100 was monitored by adding trace amounts of ^{45}Ca; it chelated nearly 100% of the ^{45}Ca as determined by scintillation counting.

1 ml of chicken precipitate fraction, pH 5.2, was dialyzed against 1 l of 0.5 mM EGTA, 20 mM Tris · HCl, pH 7.4, at 4°C. After 6 h, the dialysis tubing was transferred to a polyethylene flask containing 2 l of 20 mM Tris · HCl, pH 7.4, and dialysis was continued for 15 h with two changes of buffer to remove EGTA. The first step of the phosphorylase kinase assay was performed in disposable plastic culture tubes. The concentrations of Ca^{2+} in the Ca^{2+}-free phosphorylase kinase reaction mixture, determined by atomic absorption spectroscopy, was 11 μM as compared to 240 μM in the usual, untreated phosphorylase kinase reaction mixture.

Materials

Enzymes and cofactors were purchased from Sigma Chemical Co., St Louis, Mo.; Tris and sucrose from Schwartz/Mann, Orangeburg, N.Y.; ultrapure MgO from J.T. Baker Chem. Co. (Ultrex), Phillipsburg, N.J.; Chelex 100 (200—400 mesh) from Bio-Rad Laboratories, Richmond, Calif.; ATP-γ-S (adenosine 5′-*O*-3-thiotriphosphate) from Boehringer Mannheim Corp., San Francisco, Calif.

Results

Time course of phosphorylase kinase assay

The time course of the phosphorylase kinase assay using endogenous or exogenous phosphorylase *b* is shown in Fig. 1. The comparative rates of the reaction using either ATP or ATP-γ-S are also shown. In agreement with findings of Gratecos and Fischer [14] on the conversion of skeletal muscle phosphorylase *b* to *a* [14], the rate of conversion with ATP-γ-S proceeded at about one-fifth the rate obtained with ATP (Fig. 1A). The conversion of

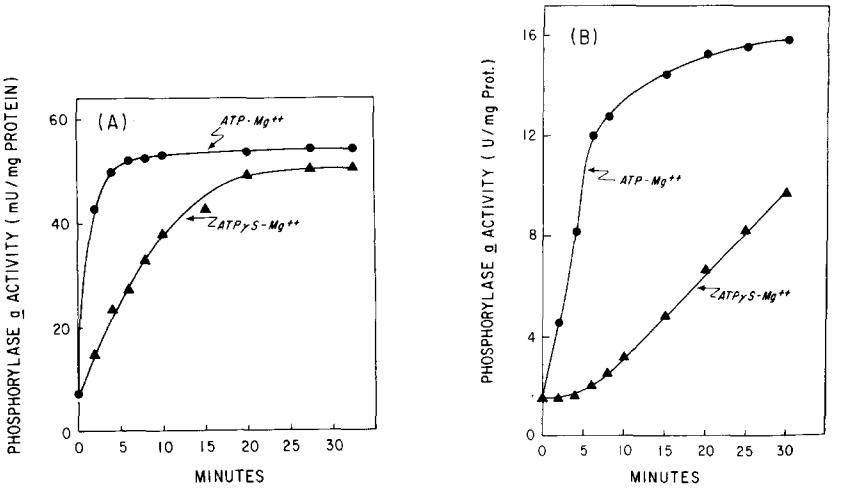

Fig. 1. Time course of conversion of phosphorylase *b* to phosphorylase *a* by phosphorylase kinase. (A) endogenous phosphorylase *b* as substrate (using 0.22 mg of pH 5.2 precipitate fraction per 50-μl assay). (B) exogenous rabbit skeletal muscle phosphorylase *b* as substrate (240 units/ml) using 0.11 mg of pH 5.2 precipitate fraction per assay. Phosphorylase kinase activity was expressed as milliunits/mg protein in panel A and units/mg protein in panel B. Either 1 mM ATP (●) or 1 mM ATP-γ-S (▲), was used as cofactor. Phosphorylase kinase assay were carried out as described in detail under Materials and Methods.

endogenous phosphorylase *b* to *a* was virtually complete in 5 min with ATP. With ATP-γ-S, conversion of phosphorylase *b* to *a* appeared to be complete at 25 min. The time course was nearly linear up to 10 min with ATP-γ-S.

The rates of the reaction using ATP or ATP-γ-S with exogenous phosphorylase *b* were also studied. With ATP, the rate was linear for 5 min which was the time routinely used for assay. With ATP-γ-S, there was an initial lag phase but the reaction was linear from 10 to 30 min. The lag phase could be reduced by using more pH 5.2 precipitate fraction (see Fig. 4B). This was in contrast to the utilization of endogenous phosphorylase *b* as substrate where no lag phase was observed (Fig. 1A).

ATP-γ-S was chosen because it was reported by Gratecos and Fischer [14] that the thiophosphorylase *a* produced is resistant to phosphorylase phosphatase [4]. Moreover, the slower rate of the reaction made it possible to

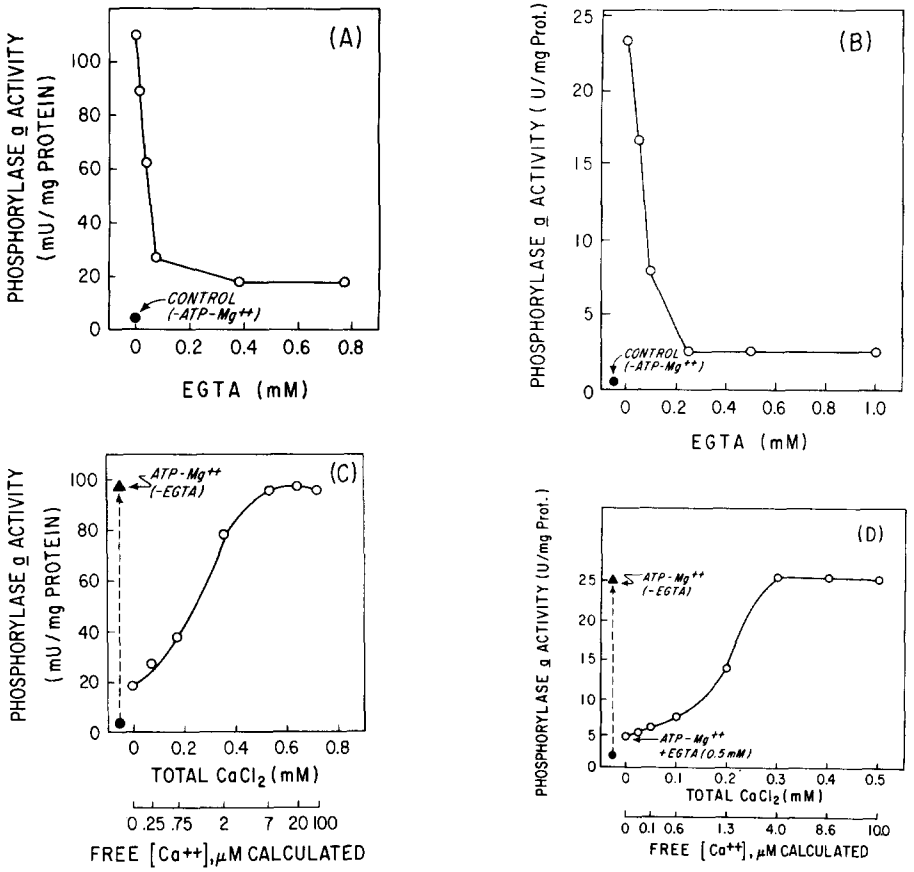

Fig. 2. Effects of EGTA and Ca^{2+} on phosphorylase kinase activity. Phosphorylase kinase reaction mixture in all cases were incubated for 5 min at 30°C. (A) inhibition of activation of endogenous phosphorylase *b* (B) inhibition of activation of exogenous (rabbit skeletal muscle) phosphorylase *b* (273 units/ml). (C) reversal of EGTA inhibition of activation of endogenous phosphorylase *b* by addition of $CaCl_2$ (0.5 mM EGTA). (D) reversal of EGTA inhibition of exogenous phosphorylase *b* (264 units/ml) by addition of $CaCl_2$. The lower abscissa of panel C and D show the calculated concentration of free Ca^{2+} based on the dissociation constant of $1.3 \cdot 10^{-6}$ M for Ca^{2+}-EGTA complexes [15]. Solid symbol (●) indicates the basal phosphorylase activity.

determine the initial linear rate more easily, particularly in studies utilizing endogenous phosphorylase *b*.

Effects of EGTA and Ca^{2+} on phosphorylase kinase activity

EGTA inhibited the phosphorylase kinase activity of chicken adipose tissue markedly. This was true either with endogenous or exogenous phosphorylase *b* as substrate (Figs. 2A and 2B). Half maximum inhibition was observed at 0.05 mM and maximal inhibition, about 80—90%, at approx. 0.2 mM. EGTA at the highest concentration used (1 mM) did not itself interfere with the assay of total endogenous phosphorylase activity (assayed in the presence of 2 mM AMP).

The inhibition of phosphorylase activation due to EGTA was prevented by adding $CaCl_2$. The maximum drop was less than 0.1 pH unit when 0.5 mM $CaCl_2$ was added to 0.5 mM EGTA. Again, the experiments were performed both with endogenous and exogenous substrates as shown in Figs. 2C and 2D. Full activation was achieved at 0.3 mM $CaCl_2$ in the presence of 0.5 mM EGTA. Since EGTA selectively binds Ca^{2+}, the amount of free Ca^{2+} in the titrated reaction mixture must be at the micromolar level. The lower abscissa shows the calculated free Ca^{2+} concentration using a dissociation constant of $1.3 \cdot 10^{-6}$ M as reported by Portzehl et al. [15]. The estimated free Ca^{2+} concentrations needed for maximum activation were 7 and 4 μM, respectively, for endogenous and exogenous phosphorylase *b* (Figs. 2C and 2D). The small increase in sensitivity to Ca^{2+} when exogenous phosphorylase *b* was used as substrate was observed consistently. This could be due to contamination of minute amounts of Ca^{2+} in the exogenous phosphorylase *b* preparations. However, the free Ca^{2+} values estimated to be in equilibrium with the Ca^{2+}-EGTA complexes can only be taken as approximations since the true dissociation constant in the reaction mixture is not known with certainty.

Phosphorylase phosphatase

To test whether the effects of EGTA and Ca^{2+} might be on phosphorylase phosphatase, its activity was measured in the pH 5.2 precipitate fraction using an excess of purified skeletal muscle phosphorylase *a* as substrate. The time course of the reaction and its inhibition by the combination of 50 mM β-glycerophosphate and 10 mM KF is shown in Fig. 3. KF at 10 mM, a level that did not inhibit phosphorylase kinase activity, inhibited phosphorylase phosphatase by 45%. β-Glycerophosphate alone at 50 mM inhibited the phosphorylase phosphatase by 66%. The combination of the two substances inhibited phosphorylase phosphatase by 75% (Fig. 3). The rate of the phosphatase reaction was very slow compared to that of the kinase reaction. At 5 min, which was routinely used for phosphorylase kinase assays, the changes due to phosphorylase phosphatase were very small and even these changes were completely inhibited by the combination of 10 mM KF and 50 mM β-glycerophosphate used routinely in the standard phosphorylase kinase assay mixture.

Reversibility of EGTA inhibition

The results described above showed that Ca^{2+} could prevent EGTA inhibition when Ca^{2+} and EGTA were added simultaneously but did not establish

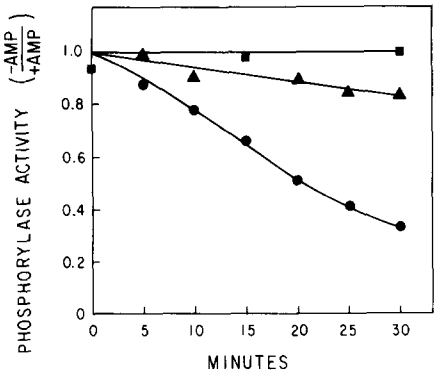

Fig. 3. Inhibition of phosphorylase phosphatase by KF and β-glycerophosphate. Purified phosphorylase *a* (270 units/ml) from rabbit skeletal muscle was used as substrate. ■, control incubation of phosphorylase alone; ●, phosphorylase *a* plus pH 5.2 precipitate fraction (0.16 mg); ▲, as above with additions of β-glycerophosphate (50 mM) and KF (10 mM).

whether or not the inhibition by EGTA was a readily reversible process. As shown in Fig. 4A, using endogenous phosphorylase *b* as substrate, the time course of activition was quite linear up to 10 min; the activation process was stopped immediately and completely on addition of EGTA at 3 min. This inhibition was promptly reversed by the addition of 0.5 mM $CaCl_2$ at 15 min. Similar results were obtained using exogenous phosphorylase *b* as substrate. Again, there was an initial lag phase (see Fig. 1B) but the time course was

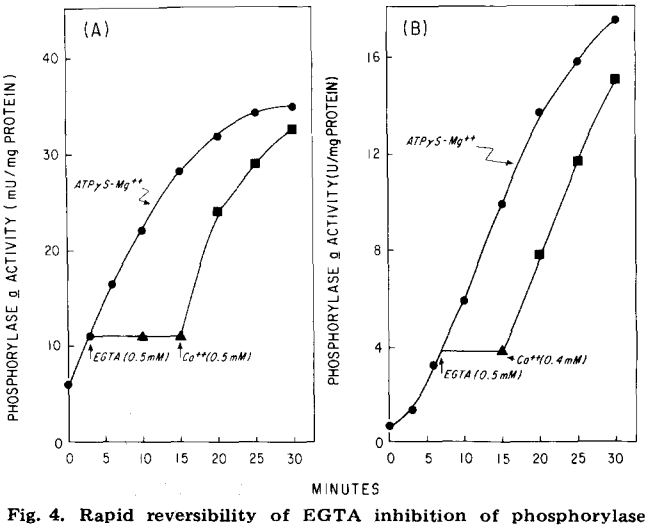

Fig. 4. Rapid reversibility of EGTA inhibition of phosphorylase kinase by Ca^{2+}. (A) time course of phosphorylase activation with endogenous phosphorylase *b* using 1 mM ATP-γ-S (●). At 4 min, an aliquot of 200 μl was transferred to a tube containing EGTA to give a final concentration of 0.5 mM. Phosphorylase *a* was assayed at 10 and 15 min (▲). At 15 min, an aliquot of 50 μl from this reaction mixture with EGTA added was transferred to a tube containing $CaCl_2$ to give a final Ca^{2+} concentration of 0.5 mM. Aliquots of 5 μl were then assayed for phosphorylase *a* activity at 20, 25 and 30 min (■). (B) exogenous phosphorylase *b* from rabbit skeletal muscle (220 units/ml) was used as substrate with the addition of 0.16 mg pH 5.2 precipitate fraction per assay. Aliquots of 5 μl were taken for assay as described under Materials and Methods at the time intervals indicated. Symbols as for panel A.

nearly linear from 5 to 30 min. Addition of 0.5 mM EGTA at 6 min blocked the activation process completely. When 0.4 mM Ca^{2+} was added at 15 min, the inhibition was immediately reversed without a lag (Fig. 4B).

Direct demonstration of Ca^{2+} requirement for phosphorylase kinase activity

Attempts were made to investigate whether phosphorylase kinase activity in chicken adipose tissue could be stimulated by Ca^{2+} directly, that is, without the use of EGTA. This was done by using Ca^{2+}-free reagents throughout and using enzyme fractions previously dialyzed for 6 h against 0.5 mM EGTA. As shown in Fig. 5, the enzyme previously dialyzed against 0.5 mM EGTA showed substantial phosphorylase kinase activity in the absence of added Ca^{2+}, the phosphorylase *a* activity increasing from basal value of 1.3 to 16.1 milliunits/mg protein. The phosphorylase activity ratio only rose to 0.41 in contrast to results with untreated pH 5.2 precipitate fraction, in which case the activity ratios rose to values nearer 1.0. Thus, in contrast to the pH 5.2 precipitate fraction, in which addition of EGTA almost completely inhibited phosphorylase kinase activity, the fraction dialyzed previously against EGTA was not so inhibited. Nevertheless, as shown in Fig. 5, the addition of micromolar concen-

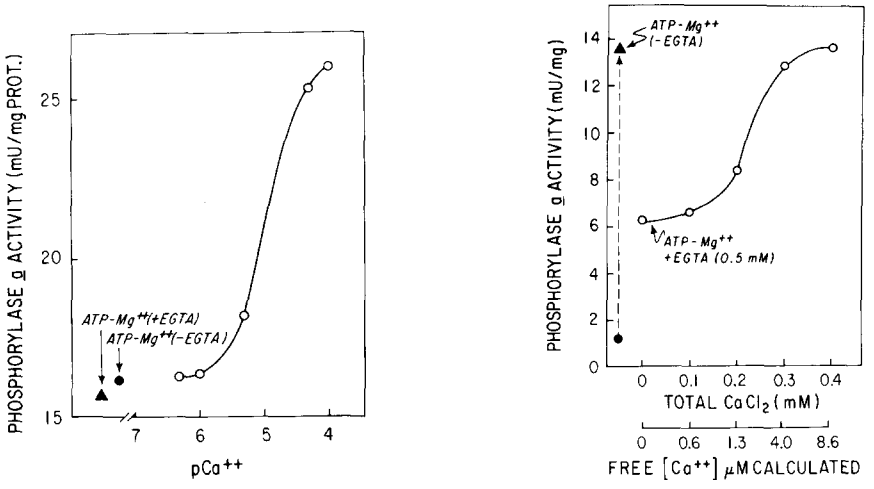

Fig. 5. Direct demonstration of Ca^{2+}-dependent activation of phosphorylase kinase using Ca^{2+}-free reagents. Reagents were depleted of Ca^{2+} by treatment with Chelex 100. Enzyme fractions were dialyzed against EGTA (0.5 mM) for 6 h, followed by two changes of Ca^{2+}-free Tris · HCl buffer, pH 7.4, for 16 h. Experiments were carried out in plastic test tubes with the addition of 0.18 mg of dialyzed pH 5.2 precipitate fraction per assay Concentrations of $CaCl_2$ were added directly in the absence of EGTA (○). Conversion of endogenous phosphorylase *b* to *a* was also carried out without addition of $CaCl_2$ using ATP-Mg^{2+} (●) or ATP-Mg^{2+} in the presence of 0.5 mM EGTA (▲).

Fig. 6. Reversal of EGTA inhibition of rat adipocyte phosphorylase kinase by Ca^{2+}. Conditions were similar to those described in Fig. 2C except that the source of phosphorylase kinase and endogenous phosphorylase *b* was the pH 5.2 precipitate fraction of rat adipocytes (0.09 mg protein per assay). The endogenous phosphorylase *b* with the activity ratio of 0.07 (●) was converted to *a* at pH 6.8 in the presence of 5 mM Mg^{2+}, 1 mM ATP and creatine kinase ATP-regenerating system (▲). The conversion of phosphorylase *b* to *a* was partially inhibited by EGTA (0.5 mM). Concentrations of $CaCl_2$ as indicated were added to reverse the inhibition due to EGTA (○). The lower abscissa shows the calculated concentration of free Ca^{2+} based on the dissociation constant of $1.3 \cdot 10^{-6}$ M for Ca^{2+}-EGTA complexes [15].

trations of Ca^{2+} further stimulated phosphorylase kinase activity in the absence of EGTA. A 50% maximal stimulation was obtained at 10 μM and maximal stimulation at approx. 100 μM. As discussed further below, it appears that the extensive dialysis against EGTA necessary to free the system from Ca^{2+} somehow modifies the phosphorylase kinase but it is clear nonetheless that preparations so treated show a definite stimulation by micromolar concentrations of Ca^{2+}.

Effects of EGTA and Ca^{2+} on phosphorylase kinase activity of rat adipocytes

The Ca^{2+} requirement of phosphorylase kinase from rat adipocytes was reinvestigated using endogenous phosphorylase *b* as substrate [7]. As shown in Fig. 6, 0.5 mM EGTA inhibited the conversion of phosphorylase *b* to *a* by 60%. This inhibition was reversed by addition of Ca^{2+} and maximal activation was obtained at approx. 0.4 mM Ca^{2+}, corresponding to a calculated free Ca^{2+} concentration of about 8.6 μM.

Discussion

The results reported above clearly establish that adipose tissue phosphorylase kinase requires micromolar concentrations of free Ca^{2+} for optimal activity. The enzyme from chicken adipose tissue was almost without activity in the presence of added EGTA; the enzyme from rat adipocytes had some activity against endogenous phosphorylase *b* even in the presence of EGTA but it showed clear stimulation on addition of micromolar concentrations of Ca^{2+}. Furthermore, as previously noted, the activation of exogenous phosphorylase *b* was inhibited 60% by EGTA [7]. The apparent Ca^{2+}-independent activation of endogenous phosphorylase previously reported may have been due to the longer incubation time used. Thus, the difference in regard to inhibition by EGTA between the phosphorylase kinase from adipose tissues of the two species is one of degree.

Although Ca^{2+} reversed the inhibitory effects of EGTA, it remained a possibility that the inhibition and reversal were not directly attributable to Ca^{2+}. For example, EGTA (but not EGTA-Ca^{2+}) might directly inhibit the enzyme or Ca^{2+} might release from EGTA some other divalent cation needed for phosphorylase kinase activity. Therefore, experiments were performed in the absence of EGTA to demonstrate the Ca^{2+} effect directly. For this purpose, it was necessary to dialyze the enzyme fraction against EGTA to remove endogenous Ca^{2+}. This dialysis against EGTA, if prolonged, resulted in complete inactivation of phosphorylase kinase while the activity of the endogenous phosphorylase was not affected. On the other hand, dialysis against buffer without EGTA for as long as 2 days did not inactivate phosphorylase kinase nor alter its Ca^{2+} requirement. Nevertheless, it was possible to dialyze over a shorter time interval and obtain a preparation using Ca^{2+}-free reagents that showed definite stimulation by Ca^{2+}, as shown in Fig. 5. However, this preparation showed considerable activity even in the presence of added EGTA. This was in contrast to the non-dialyzed preparations which showed almost complete dependence on added Ca^{2+} in the presence of EGTA. These results suggest that the dialysis against EGTA (but not against buffer not containing EGTA)

converts the phosphorylase kinase to an alternative form not fully dependent upon addition of Ca^{2+}.

The concentration of Ca^{2+} needed to stimulate the enzyme prepared using Ca^{2+}-free reagents was higher than the calculated concentration needed in experiments utilizing Ca^{2+}-EGTA complexes. One likely explanation for this is that the pH 5.2 precipitate fraction contains a number of other proteins (and also lipids) that bind some Ca^{2+}. Randle and coworkers [16] also reported that the Ca^{2+} concentration required to activate purified pig heart pyruvate dehydrogenase phosphate phosphatase using Ca^{2+}-free reagents was some 50 times greater than the calculated Ca^{2+} concentration required when using Ca^{2+}-EGTA buffers.

The possibility that the reciprocal effects of EGTA and Ca^{2+} might be on phosphorylase phosphatase rather than on phosphorylase kinase was considered. An example of this type of alternative mechanism has been reported by Haschke et al. [17] in their studies on the intact protein-glycogen complex isolated from rabbit skeletal muscle. Phosphorylase phosphatase in this system was reversibly inhibited in the presence of ATP-Mg^{2+} and Ca^{2+} during "flash activation". In the present studies, the inclusion of KF and β-glycerophosphate during the first step of the phosphorylase kinase assay effectively inhibited the action of phosphorylase phosphatase. Furthermore, in experiments utilizing ATP-γ-S instead of ATP it was possible to show a similar EGTA inhibition and Ca^{2+} reversal. Gratecos and Fischer [14] have shown that the thiophosphorylase *a* formed from skeletal muscle phosphorylase *b* in the presence of ATP-γ-S is resistant to the action of phosphorylase phosphatase [14]. Assuming the same is true in this system the results with exogenous phosphorylase *b* of skeletal muscle described above (Fig. 4) provide further evidence that the effects of EGTA and Ca^{2+} must be on the phosphorylase kinase reaction itself.

In skeletal muscle, Ca^{2+}-dependent activation of phosphorylase kinase is believed to play an important role in linking glycogenolysis to muscular contraction [18]. The possibility that Ca^{2+} may also serve as a messenger for a variety of subcellular events in other tissues has been extensively discussed [19]. The physiological role of Ca^{2+} as related to phosphorylase kinase activation in adipose tissue and in liver [20] remains to be elucidated.

Acknowledgements

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